

ZAHNÄRZTLICHES INSTITUT DER UNIVERSITÄT ZÜRICH

Abteilung für Orale Mikrobiologie und Allgemeine Immunologie

Vorsteher: Prof. Dr. B. Guggenheim

**A RAT FIBROBLAST MODEL TO STUDY POSSIBLE IMMUNE REACTIONS
IN PERIODONTAL DISEASE IN VITRO**

INAUGURAL-DISSERTATION

zur Erlangung der Doktorwürde
der Zahnmedizin der Medizinischen Fakultät
der Universität Zürich

vorgelegt von

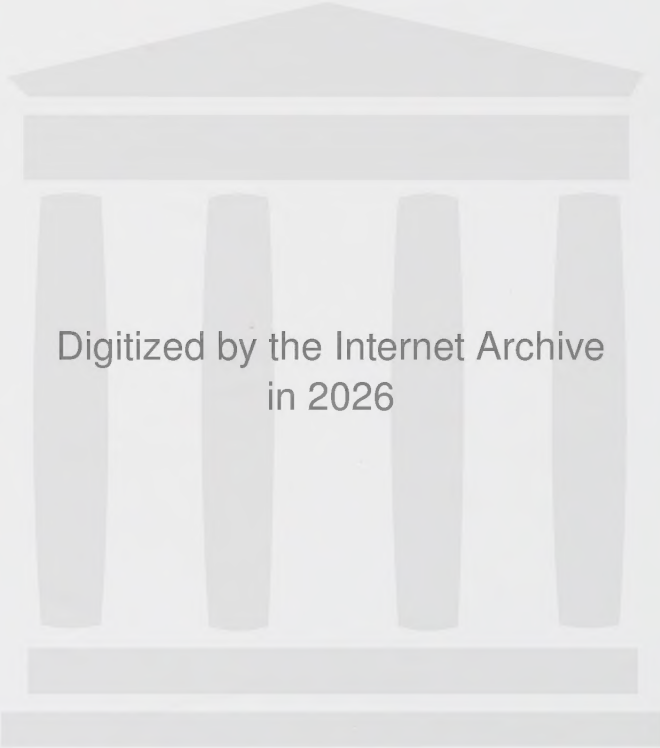
ARTHUR HEFTI

von Schwanden (Kt. Glarus) und Zürich

Genehmigt auf Antrag von Prof. Dr. B. Guggenheim

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1. General Introduction

The immune system serves to recognize and dispose of foreign matter or altered self-structures formed in the body of vertebrates. Sometimes, after repeated contact with antigens, it is accompanied by violent side effects. Repeated local contact with foreign protein can, for example, lead to local tissue destruction, or an organ transplant may be rejected. Both are so called hypersensitivity reactions mediated by the immune system. It has been found that several pathomechanisms are operative in hypersensitivity reactions. According to the mechanisms, Gell, Coombs and Lachmann have distinguished the following four reaction types:

- I Anaphylaxis
- II Antibody mediated cytotoxicity
- III Antigen-antibody complex mediated hypersensitivity
- IV Cell-mediated, delayed hypersensitivity.

Inflammatory reactions in the periodontium have been repeatedly ascribed to one of these hypersensitivity types. A flood of literature has been published on this particular subject. Papers by Hulin (1929), Brandtzaeg and Kraus (1965), Ivanyi and Lehner (1970) and Horton, Leikin and Oppenheim (1972) may be considered as milestones in the evolution of changing concepts.

Sequential, morphometric analyses of the disease revealed changes in the quantitative and qualitative composition and structure of periodontal tissues (Page & Schroeder 1973 and 1976, Schroeder & Graf-deBeer 1976). According to these authors an increasing loss of collagen, beginning in perivascular regions, was characteristic of the development of the lesions. This loss began in the fibres supporting the gingival margin, reached, in a later stage, the junctional and

finally the pocket epithelium. The number of fibroblasts present remained constant until the stage of the established lesion was reached but their total volume was about three times larger, probably as a consequence of pathological alterations. Contrary to findings in rats, bone loss in humans and dogs was observed in advanced lesions only. The fate of the established lesion was uncertain. It could persist for a prolonged period or progress into an advanced lesion. There is no satisfactory explanation for the variability of the sequence of these events.

In spite of the efforts invested into these impressive investigations, they were all based on speculation, within the limits of existing theories of the causal pathomechanisms. Progress in the understanding of the exact pathomechanisms involved in the breakdown of connective tissue was thought to lay in more dynamic experimentation. Based on a gnotobiotic rat model (Guggenheim & Schroeder 1974) a cytotoxicity test system was established with the goal to test in vitro possible mechanisms causing injury to fibroblasts. This problem was approached in the following manner:

1. A cytotoxicity model with fibroblasts of the above mentioned rat strain was established. Culture conditions in particular were thoroughly investigated.
2. The direct cytotoxicity of fractions from Actinomyces viscosus Ny 1 was measured. A. viscosus was originally isolated from hamsters by Jordan and Keyes (1964) and was shown to be the aetiologic agent of periodontal disease in rodents by Jordan, Fitzgerald and Stanley (1965).
3. The cytotoxicity mediated by antibodies against A. viscosus Ny 1 and complement was assessed.
4. The activation of the complement sequence by fractions of A. viscosus Ny 1 in the absence of antibody by the alter-

native pathway and the resulting cytotoxic effect were investigated. In particular the effect of the third complement component (C3a) was investigated in some detail.

2. The establishment of culture conditions for a cytotoxicity model with rat fibroblasts.

2.1. Introduction.

The first successful experiment in tissue culture was carried out by R.G. Harrison (1907). He tried to obtain a method by which the end of a growing nerve could be directly observed. Even in his best experiments, however, the explants died after a few weeks. A. Carrel (1912) developed new techniques which allowed him to solve the problem of "indefinite life" of cells isolated from mammalian but not human tissues. The cells were fed with coagulated plasma. The next step was to modify the culture conditions to allow the continued proliferation of single cells. Sanford, Earle and Likely (1948) established a clone culture of sarcoma producing cells from C3H mice. In 1952 Gey, Coffman and Kubicek published a short communication on the proliferative capacity of human cervical carcinoma and normal epithelium in vitro. It was the first time that cells from human tissue survived in tissue culture.

Eagle (1955) introduced a medium containing a mixture of amino acids, vitamins, cofactors, carbohydrates and salts, supplemented with 1% of dialyzed horse or human serum. A milestone in tissue culture was set by Hayflick and Moorhead (1961). They described and characterized the development of 25 cell lines of human origin and suggested the use of diploid human cells for human virus vaccine production.

In recent years, tissue cultures were used for toxicity tests (Hausmann 1974, Metcalfe 1971, Ruddle & Waksman 1968). In oral biology, tissue cultures were introduced by Raisz (1965) to study effects of different agents on cultured bone. Extracts of human dental plaque were shown to be cytotoxic

to several cultured mammalian cell lines (Baboolal, Mlinek & Powell 1970, Levine, Adams & Cowley 1973, Mlinek, Buchner, Hennig & Begleiter 1974).

In this part of the study experiments were carried out to determine the optimal culture conditions for fetal rat fibroblasts. It was therefore necessary to investigate the effect of various serum concentrations and seeding densities on the plating efficiency, saturation density and division rate of rat fibroblasts.

2.2. Material and Methods.

Nutritional medium (NM): The medium used was Dulbecco's modification of Eagle's minimal essential medium (MEM, Seromed, Munich, GFR), supplemented with 10, 15 or 20% heat-inactivated fetal calf serum (FCS), 1% of 100x concentrated non-essential and essential amino acids (Seromed), penicillin G (Hoechst) and streptomycin (Novo) both 100 µg/ml. To maintain the pH at 7.0 cultures were incubated in an atmosphere of air supplemented with 5% CO₂.

The cells were cultured in disposable plastic flasks (Costar tissue culture flasks 3025 & 3075) and for experimental purposes were subsequently transferred to petri dishes (Nunc N-1420, Ø 35 mm).

Primary cultures: Cultures of 18-20 day old rat fetuses (RIC Sprague-Dawley, originally provided by the Tierfarm AG, Siseln, Switzerland) were prepared by fine mincing the skin with a pair of tweezers. Cells were then disaggregated in a phosphate buffered saline-0.25% trypsin solution for 90 min on a magnetic stirrer at room temperature. The trypsin solution was then removed by centrifugation in a cooled Sorval centrifuge (800 g, 10 min) and the cells washed twice in Hanks' balanced

salt solution. Afterwards the cells were suspended in nutritional medium. Forty-eight hours later, confluent monolayers had formed.

Subcultivation procedures: As soon as the cells were fully sheeted they were subcultivated regularly every 48 h. For this purpose the medium was removed from the flasks and 2 ml of a trypsin-ethylendiamino-tetra-aceticacid solution (0.025% trypsin, 0.016% EDTA-Na-salt) was added until the cells began to contract. At this moment the trypsin solution was carefully removed by suction and the flask was then incubated for 10 min at 37 C. The cells were detached thereafter in 5 ml of NM by gentle agitation. Another 5 ml of NM was added and the cell aggregates were agitated until a suspension of single cells was obtained. The suspension was then equally distributed into 2 new flasks.

Preservation and storage of living cells: Deep freezing was chosen as the most convenient method to obtain a stock of standardized cells. Flasks containing healthy cells in the 4th passage, still in the growing phase, were selected. The cells were removed by trypsinization as described above and suspended at a concentration of 5×10^6 cells/ml in NM containing 20% FCS and 5% dimethyl sulfoxide.

The cells of all cultures to be frozen were pooled in one flask and dispensed in 1 ml portions into 5 ml glass ampules which were sealed in an oxygen flame. The ampules were wrapped in cellulose, packed in small ampule-containers (Nunc No N-247) and cooled down to -25 C at a rate of 1 C per minute. Finally they were transferred to an ultra deep freezer (Revco ULT 1785) and stored at -80 C until used.

For recovering frozen cells, the ampules were immediately plunged into a 37 C water bath. As soon as the content had thawed, the suspension was pipetted into a tissue culture

flask, washed with 5 ml of Hanks' solution, resuspended in fresh NM and distributed to tissue culture flasks with a culturing surface of 25 cm^2 at a concentration of 2.5×10^6 cells/ml.

Culture conditions for evaluating growth parameters: Cells originating from the seventh to tenth passage were used to perform the experiments to be described.

Plating efficiency (PE): 2×10^2 cells, suspended in 2 ml NM supplemented with either 10 or 15% FCS, were added to a petri dish and incubated at 37 C in an atmosphere of 5% CO_2 /95% air for ten days. After that, the cells were fixed in a formaldehyde-glutaraldehyde solution (4% paraformaldehyde, 5% glutaraldehyde, 0.01 M Na-cacodylate) for 1 h and stained for 15 min with Giemsa diluted 1:10 in distilled water. PE was calculated as the number of colonies grown per 100 inoculated cells.

Saturation density, generation time and division rate: Suspensions containing 10^3 , 10^4 or 10^5 cells/ml were prepared in NM, supplemented with 10, 15 or 20% FCS. Two ml of each were plated in small petri dishes with a surface of 10 cm^2 and incubated at 37 C in 5% CO_2 /95% air. This corresponds to 10^2 , 10^3 and 10^4 cells/ cm^2 . Every second day until day 10 at least 2 cultures of each treatment were trypsinized in 2 ml of a 0.025% trypsin solution. Then the cells were resuspended with a pipette until a homogeneous distribution was achieved. Thereafter the cell number was estimated in an electronic cell counter (Coulter Modell ZB_T).

2.3. Results.

Growth in tissue cultures does not occur when the cell density of the inoculum falls below a critical level (Eagle &

Piez 1962). On the other hand growth occurs in a fairly wide range of cell concentrations. Experiments in which the relationship between cell density of the inoculum and resulting division rate were studied are shown in Figures 1, 2 and 3. Cells were inoculated in 3 different dilutions and were counted in 48 h intervals up to 10 days. In the same intervals, the medium was changed in the remaining cultures. The mean number of cells obtained after a determined period was dependent on the size of the inoculum, but even with an initial cell density of 10^3 cells/ml, growth was initiated. The division rate was calculated following the equation:

$$\text{division rate} = \frac{\log x_1 - \log x_0}{(t_1 - t_0) \log 2}$$

With an initial cell density of 10^3 cells/ml the maximum division rate was reached between day 6 and 8 with 10% and 15% FCS supplement respectively. With 20% FCS supplement the maximum value was registred between day 4 and 6. With an initial cell density of 10^4 cells/ml and 10^5 cells/ml respectively the highest division rates were registred between day 2 and 4 and were independent on the amount of serum supplemented to the NM.

The composition of the culture medium is of great importance in tissue culture. Above all the addition of serum influences the growth kinetics as evidenced by the division rate. In the following, cells from monolayers were plated at concentrations of 10^3 , 10^4 or 10^5 cells/ml respectively. For each serum concentration and seeding density the mean cell number of at least 2 plates was calculated at 48 h intervals. The highest increase as well the highest value for the division

Table I

The effect of the serum concentration and seeding density on the generation time of fetal rat cells. The cells originated from the seventh to tenth passage.

Seeding density cells/cm ²	Period of observation days	Generation time (hours) for different serum concentrations		
		10%	15%	20%
10 ²	0 - 2	56.7	23.6	44.8
	2 - 4	27.3	23.6	27.6
	4 - 6	26.9	21.2	17.9
	6 - 8	19.5	19.8	27.6
	8 -10	23.1	51.3	40.3
10 ³	0 - 2	61.4	21.7	144.9
	2 - 4	21.0	21.9	16.4
	4 - 6	28.6	36.1	27.9
	6 - 8	29.7	67.6	41.8
	8 -10	103.1	625.0	114.9
10 ⁴	0 - 2	44.0		62.2
	2 - 4	38.8		20.6
	4 - 6	46.1		90.0
	6 - 8	324.4		----
	8 -10	----		138.2

rate was obtained with a 20% serum supplement (Fig.3). When 15% FCS was added the division rate behaved quite differently: no phase of increasing division rate was observed. Up to day 4 the division rate remained relatively constant (Fig.2). The generation time (GT) was calculated as reciprocal value of the division rate and represents a mean value for each 48 h interval. Table I shows the effect of the serum concentration and the seeding density on the GT. The seeding density had little effect on the minimum GT, the fastest reproductions were observed in cultures seeded with either 10^3 or 10^4 cells/ml. The longest GT was calculated between day 8 and 10 for a 15% FCS supplement and an initial seeding density of 10^4 cells/ml, indicating practically no increase in the cell number (Table I). With a seeding density of 10^5 cells/ml a decrease of the absolute cell number was observed between day 8 and 10 with a serum supplement of 10% and between day 6 and 8 with a serum supplement of 20% (Table II). The saturation density (SD) is defined as the highest cell number per cm^2 to which a given cell strain or line is capable of packing. SD may be furthermore characterized by a division rate tending towards 0, or by a stable cell number despite of the cultures being regularly fed with fresh NM. In Table II the effect of either the seeding density or the serum concentration on the SD in function of the time is shown. With a seeding density of 10^3 cells/ml SD was not attained within 10 days. With an inoculation density of 10^4 cells/ml SD was reached at day 10 when the cultures were supplemented with 15% FCS. The respective GT for the period day 8 to 10 was 625 h, indicating a nearby growth stop. With 10^5 cells/ml SD was attained by day 6 to 8 independent of the amount of serum supplemented. The value for the SD varied between 9.5×10^5 cells/ cm^2 and 12.9×10^5

Table II

The effect of the serum concentration and the seeding density on the cell density in cultures of fetal rat cells. The cells originated from the seventh to tenth passage.

Seeding density cells/cm ²	Period of observation days	Cell density (cells/cm ²) for different serum concentrations			
		10%	15%	20%	
10 ²	0 - 2	187 ± 42	426 ± 10	218 ± 21	
	2 - 4	634 ± 125	1746 ± 21	728 ± 229	
	4 - 6	2183 ± 166	8388 ± 135	4667 ± 457	
	6 - 8	11994 ± 468	44974 ± 883	15580 ± 956	
	8 -10	50555 ± 520	86040 ± 5051	35557 ± 624	
10 ³	0 - 2	1788 ± 146	4802 ± 229	1310 ± 166	
	2 - 4	8697 ± 2266	21972 ± 135	9884 ± 364	
	4 - 6	27793 ± 4043	55149 ± 10165	32564 ± 2671	
	6 - 8	85177 ± 5051	90125 ± 4365	72299 ± 5851	
	8 -10	117439 ± 1174	94968 ± 1414	96444 ± 738	
10 ⁴	0 - 2	22139 ± 332		17773 ± 1039	
	2 - 4	52166 ± 2619		89387 ± 1247	
	4 - 6	107316 ± 3201		129299 ± 416	
	6 - 8	118905 ± 3368		98533 ± 10186	

cells/cm² (Table II).

The plating efficiency (PE) is the number of cells that develop into colonies per 100 cells inoculated, when the inoculum is sparse enough to permit growth of separate colonies. PE-tests were performed with 2 serum concentrations. With a 10% FCS supplement to the medium 6.7 ± 0.8 colonies were counted per 100 cells inoculated. Feeding with 15% FCS resulted in a PE of 15.4 ± 1.7 .

2.4. Discussion.

The present data demonstrate that conditions suitable for tissue culture of fetal RIC rat cells were established. Criteria to evaluate optimum growth conditions were the following parameters: saturation density, division rate (generation time) and plating efficiency. The serum concentration of the NM and the size of the inoculum had a major effect on these parameters. Most of the normal cell lines have a constant SD. With time, SD increases, indicating a progressive loss of contact inhibition characteristic of normal cells (Todaro & Green 1963). Contact inhibition is a property of normal cells (Abercrombie & Heaysman 1953, Aaronson & Todaro 1968) which is lost to some degree by malignant and most established cell lines (Todaro & Green 1963). Most experiments so far described were performed with cells of the 7th passage and were therefore believed to be normal cell lines, which should, according to above, show a constant SD. With progressive passages a shift to higher saturation densities was observed. From passage 20 onwards, a failure of contact inhibition was sometimes observed. Occasionally, a few passages later, the cell sheet peeled off, indicating a complete loss of contact inhibition. Within the early cell passages used in the present experiments,

the SD was rather constant. Holley and Kiernan (1968) showed with 3T3 cells that cessation of growth is independent of contact inhibition but depends on exhaustion of an essential growth factor when cells become crowded. Our results differ from those cited above because the SD was largely independent of the serum concentration in the medium. The discrepancy is most likely due to different medium composition and culture procedures. Holley and Kiernan (1968) used Vogt and Dulbecco's modification of Eagle's MEM, but without addition of vitamins, essential and non-essential amino acids. It is possible, that the nutritional requirements for fetal rat cells are different from those of 3T3 cells and that a factor affecting contact inhibition was among the medium additives. Another possible interpretation is that the frequent (48 h) replacement of the medium maintained a contact inhibition controlling factor at a minimum concentration.

The hypothesis was also tested whether a single addition of 10% FCS to the medium was sufficient to support cell activity, i.e. cell division and uridine uptake, for two or more days (data not shown). It was evident that independent of the inoculation density, the highest division rate was between day 1 and 2, and concomitantly the highest uridine uptake per cell was measured on day 2. It is therefore suggested that, in the culture system described, the serum concentration was not a limiting factor for the cell number at saturation density.

Ceccarini and Eagle (1971) tested the effect of pH modifications on contact inhibition and growth. They defined contact inhibition as an almost complete cessation of growth, even if the medium was changed daily. Under these circumstances the inhibition of growth ultimately observed was not deter-

mined by either nutritional deficiencies or accumulation of toxic factors. They showed furthermore that the SD was pH dependent and was very constant at optimum pH values. No precise measurements were done to determine the pH of the medium during these experiments. The pH value was estimated using phenol red as an indicator. It is suggested that deviations of the SD noticed were due to minor pH alterations. With regard to the division rate or GT for fetal rat cells, it was evident that the serum concentration had a strong influence. In addition it was obvious that parameters were independent of the size of the inoculum, if it was considerably below the saturation density. Although the medium was changed every second day in our cultures, a growth factor in serum was the limiting factor in the division rate. The division rate increased with higher serum concentrations in the range of 10% to 20%. The maximum division rate reached in the present experiments was furthermore dependent on the seeding density (Figures 1-3). This points to the existence of a growth promoting factor synthesized by these cells. Colonies from single cells may be obtained, in analogy to standard methods in microbiology, by sparse seeding of cells, which is the procedure to estimate the plating efficiency. A low plating efficiency reflects the difficulty of cell division in the absence of cell to cell contact. This does not necessarily mean that cells are unable to grow, however it reflects their inability to divide (Todaro & Green 1963). A given medium or serum may give a high PE with some cell strains and not with others and a particular medium or serum may have a very low plating efficiency but is still able to support the division of the cells to form monolayers. In general, the value for the plating efficiency is about 10% for freshly isolated, normal cells but increases to 70% when

the cells originate from established cell lines or from transformed cells (Halle 1976). The plating efficiency experiment was chosen to characterize the status of the cell lines used, i.e. to test whether it was established or not. The low values obtained with fetal rat cells were in agreement with extensive studies of other authors (Eagle & Piez 1962) who compared the state of transformation of different cell lines. For the cytotoxicity experiments to be described a serum concentration of 15% and a seeding density of 10^4 /ml was chosen. Although these conditions were not optimal, practically they best met with our needs.

3. In vitro cytotoxicity of fractions of Actinomyces viscosus Ny 1 for rat fibroblasts.

3.1. Introduction

The accumulation of bacterial dental plaque at the gingival margin leads, within a few days, to macroscopic and microscopic alterations in the gingival tissues. Perivascular collagen loss has already been measured in the initial lesion (Page & Schroeder 1973). Subsequently in the early lesion, damaged fibroblasts were observed in addition to the progressive destruction of the collagen network (Page & Schroeder 1976). Delayed hypersensitivity reactions were suggested as causative mechanisms (Horton & Mergenhagen 1974, Lehner 1977), however, a direct toxic effect of soluble plaque components on fibroblasts could, at the time, scarcely be excluded. On the contrary, in various studies (Baboolal, Mlinek & Powell 1970, Levine, Adams & Cowley 1973) a cytopathic effect of soluble plaque extracts on mammalian cells was demonstrated. However, using a ^{51}Cr release assay, Engel, Schroeder and Page (1978) were not able to detect a cytopathic effect of cell homogenates from A. viscosus on human gingival fibroblasts. The disagreement among the results cited above may be due to different cytotoxicity test techniques, target cells, or challenging substances.

To measure functional disorders of membrane permeability, ^{51}Cr (Wigzell 1965) or nucleotide labels (Thelestam & Möllby 1975) have been most widely used. Thelestam and Möllby (1975) recently introduced a cytotoxicity assay of greater sensitivity using non-metabolized, low molecular weight ^{14}C -alpha amino-isobutyric acid (^{14}C -AIB).

In the present investigation this new test system, in addition

to the conventional ^3H -uridine leakage test, was applied to measure membrane function after exposure of rat fibroblasts to various bacterial fractions - specifically, the direct cytotoxicity of an extracellular and two cellular fractions of A. viscosus Ny 1.

3.2. Material and Methods.

Microorganisms and bacterial fractions: A. viscosus Ny 1 was originally isolated from dental plaque of glucose fed rats (van der Hoeven 1974). The microorganisms were grown in 5 liter batch cultures at 37 C in a defined medium (Bowden, Hardie & Fillery 1976).

The extracellular heteroglycan (ECHG) produced by A. viscosus Ny 1 was a gift of Dr. H. van der Hoeven. Its preparation and chemical composition have been described (van der Hoeven 1974). To prepare the broken cell supernatant (BCS), Actinomyces cells obtained by centrifugation (30 min, 20'000 g) were washed three times with saline and disintegrated with glass beads ($\emptyset$ 0.11 mm) in a Braun MSK cell homogenisator (B. Braun, Melsungen GFR), cooled with CO_2 . The glass beads, large fragments and the particulate fraction including cell walls were removed by two successive centrifugation steps, the first one at low speed (30 min, 20'000 g), the second one at high speed (60 min, 105'000 g). To the supernatant ammonium sulfate (53 g/100 ml) was added resulting in approximately 80% saturation. The precipitate was harvested by centrifugation, dissolved in buffered physiological saline (PBS), dialysed against the same buffer and subsequently lyophilized. All steps were carried out at 4 C. Cell walls (CW) were prepared as described by Reed (1972) and were purified by enzymatic digestion with trypsin and

ribonuclease (Sigma Chemical Co., London).

Tissue culture: Diploid fibroblasts were derived from 18 to 20 days old RIC-Sprague-Dawley rat fetuses by finely mincing the skin followed by disaggregation with 0.25% trypsin (Todaro & Green 1963).

Cultures were then maintained in Dulbecco's minimal essential medium (DMEM, Flow Laboratories Ltd., Irvine, Scotland) enriched with 15% heat-inactivated fetal calf serum (FCS, Seromed, Munich GFR).

Cells to be used for cytotoxicity tests were from the 7th to 12th transfer after removal from primary explants and were cultivated in wells in disposable sterile polystyrene trays (Costar 3524, tissue culture dishes, Digitana AG, CH-8810 Horgen, Switzerland). The cultures were incubated at 37 C in a humid atmosphere containing 5% CO₂.

The density of adherent cells was about 10⁵ cells/cm² at the start of the experiments.

Leakage test and calculation of cytotoxic effects: The labeling of the cells with ³H-uridine and ¹⁴C-AIB (New England Nuclear Chemicals GmbH, D-6072, Dreieich GFR, specific activity 5Ci/mM and 30 mCi/mM respectively) and the procedure of the standard leakage test are presented in figure 4.

The lyophilized test substances to be added were dissolved in DMEM without FCS. In order to obtain a water soluble part, the cell wall fraction was treated for 20 sec with a Branson sonifier B12 at maximum power. Afterwards, the suspension was centrifuged at high speed (105'000 g, 30 min) and the supernatant sterilized by filtration using disposable Millex filters (0.22 µm Ø, Millipore Corporation, Bedford, Massachusetts, USA). One ml of the filtrate was used for the cytotoxicity assay.

The calculation of the cytotoxic effect was based on measure-

ments of the radioactivity present in the supernatant and the activity remaining inside the fibroblasts at the end of the experimental period. To obtain a percentage value, the radioactivity measured in the supernatant multiplied by 100 was divided by the total of activity remaining in the cells plus that present in the supernatant. Spontaneous release was determined from cultures without adding any test substances. All results represent the mean $\pm$ standard deviation of three cultures.

Measurement of radioactivity: 0.1 ml samples of the culture supernatant were transferred to scintillation vials containing 10 ml of Aquasol and counted for 1 min in a LKB Wallac 81'000 liquid scintillation counter. Automatic quench correction was used. The accuracy was controlled by the internal standard method.

3.3. Results.

General: The results of the direct cytotoxicity tests are shown in tables III and IV. A fast spontaneous release of ^{14}C -AIB, as revealed by high control values, was observed. After 1 hour of incubation 46-62% of the originally incorporated activity was found in the cell supernatant. The ^3H -uridine label was more effectively retained by the cells. After 24 h 16.5-42% of the originally incorporated label was present in the medium.

Dose-response of ^{14}C -AIB release (table III): A rather weak, but linear dose dependent cytotoxicity was observed after incubation with BCS. The extracellular heteroglycan (ECHG) induced no effect at any concentration tested. The treatment with the water soluble part of the cell wall fraction caused leakage of the marker molecule at concentrations

Table III

Release of ^{14}C -alpha-aminoisobutyric acid from fetal rat fibroblast cultures treated for 1 h with water soluble fractions of A. viscosus Ny 1 in DMEM or in DMEM alone. Broken cell supernatant (BCS), extracellular heteroglycan (ECHG), cell walls (CW). Data represent means $\pm$ standard deviation of triplicates and are percentages of maximum isotope release.

DMEM control		mg of lyophilized fraction per ml		
		0.04	0.2	1.0
61.8 $\pm$ 1.4	BCS	60.3 $\pm$ 1.8	64.8 $\pm$ 1.9	72.0 $\pm$ 8.4
46.6 $\pm$ 1.8	ECHG	48.7 $\pm$ 7.0	45.3 $\pm$ 1.7	46.0 $\pm$ 0.9
51.0 $\pm$ 5.4	CW	50.6 $\pm$ 1.3	55.7 $\pm$ 5.1	73.2 $\pm$ 4.4

0.2 and 1.0 mg/ml. However, this concentration is based on the weight of the particulate substance before high speed centrifugation and is not identical with the amount dissolved in the medium.

Dose-response of ^3H -uridine release (table IV): The results obtained with the nucleotide label after 24 h exposure time were essentially the same but more marked than those described above. Even in the lowest concentration, the BCS-fraction induced a weak but significant cytotoxic effect. A slight increase of isotope release was evident with the highest concentration of ECHG (1 mg/ml) used. Lower concentrations remained within the range of marker release as measured in the controls. A strong cytotoxic action, as measured by isotope release, was induced by the soluble cell wall fractions in all concentrations tested.

Light microscopic examinations of rat fibroblast cultures: Control fibroblasts and cells exposed to Actinomyces fractions were microscopically examined. Normal cell morphology was preserved even in concentrations of fractions where a significant cytotoxic effect was measured.

3.4. Discussion

In the present study the use of ^{14}C -AIB was based on the assumption that the sensitivity of a membrane leakage test is closely related to the molecular size of the marker isotope (Henney 1973). AIB has a low molecular weight (103) and is not metabolized in mammalian cells (Christensen, Aspen & Rice 1956), features rendering it especially useful for the detection of even minor cytotoxic membrane damage. The use of ^{14}C -AIB is, however, limited to short duration experiments. AIB is actively transported out of the cell (Chri-

Table IV

Release of ^3H -uridine from fetal rat fibroblasts upon treatment for 24 h with water soluble fractions of A. viscosus Ny 1 in DMEM or in DMEM alone. Broken cell supernatant (BCS), extracellular heteroglycan (ECHG), cell walls (CW). Data represent mean $\pm$ standard deviation of triplicates and are percentages of maximum isotope release.

DMEM		mg of lyophilized fraction per ml DMEM		
control		0.04	0.2	1.0

19.3 $\pm$ 0.3	BCS	23.6 $\pm$ 3.9	40.6 $\pm$ 4.8	62.8 $\pm$ 4.6
16.5 $\pm$ 0.6	ECHG	15.9 $\pm$ 0.4	16.4 $\pm$ 0.3	24.5 $\pm$ 0.4
42.1 $\pm$ 2.2	CW	74.0 $\pm$ 4.5	90.3 $\pm$ 3.9	94.9 $\pm$ 0.3

stensen & Handlogton 1986) by the same system mediating its uptake. This explains the relatively high spontaneous release observed in my experiments.

In order to measure cytotoxic effects caused by prolonged exposure of fibroblasts to test compounds only, a second assay was introduced. Recent experiments (Thelestam & Möllby 1975) have shown, that ^3H -uridine was not incorporated into macromolecules when the multiplication of the cells was inhibited by their density. Therefore, labelling with this nucleotide was considered to be a method of adequate sensitivity. The same authors (1976) found leakage of AIB-label to be the most sensitive method for detecting membrane damage, followed by ^3H -uridine and ^{51}Cr . Furthermore, ^{51}Cr , nonspecifically bound to intracellular proteins (Bunting, Kiely & Owen 1963) covering subtle membrane defects.

Incubation of fetal rat fibroblasts with fractions of A. viscosus Ny 1 resulted in changes in membrane permeability. The cell wall fraction and the broken cell supernatant produced "functional holes" after 1 h incubation as shown by the ^{14}C -AIB-label. This effect, directly dependent upon the concentration of the test fraction, was even more distinct after 24 h incubation time. Above all, the water soluble part of the cell wall fraction proved remarkably cytotoxic.

This fact, however, contrasts with results of a recent publication by Engel, Schroeder and Page (1978). Using a functional ^{51}Cr -release test system and a microcytotoxicity assay, they were unable to demonstrate a cytotoxic effect of A. viscosus WVU/371 homogenate on fibroblasts derived from noninflamed human gingiva.

The higher sensitivity of the assay used in my study and more highly purified test fractions could account for the apparent disagreement between our findings. Thelestam and Möllby (1976)

observed very small defects in human embryonic lung fibroblasts after treatment with 1 μ M amphotericin B. However, this was observed only when using a ^{14}C -AIB leakage test, not when a ^{51}Cr assay was used. It is, therefore, possible that A. viscosus fractions induced functional "pores" which were not detectable with a ^{51}Cr cytotoxicity assay.

The extracellular heteroglycan was not cytotoxic as evaluated in the ^{14}C -AIB test system. After 24 h of incubation time however, a slightly increased release of the nucleotide label was observed with the highest concentration of this fraction, thus suggesting a weak long-term effect.

Morphologically altered cells were not demonstrable even after incubating the fibroblasts with the highest concentration of either fraction for 24 h. Light microscopic observation revealed remaining attachment of the cells to the bottom of the culture dishes and the fibroblasts retained their characteristic spindle-like shape. This contrasts with results of Baboolal, Mlinek and Powell (1970) who found Syrian Baby Hamster kidney cells altered upon incubation with plaque extracts. It is possible that these findings are due to the presence of soluble fractions from gram negative microorganisms in their crude plaque extract. The cytotoxicity of culture filtrates of E. coli for mouse fibroblasts is well established (Yoshida et al 1975).

4. Fractions of Actinomyces viscosus activate complement by the alternative pathway.

4.1. Introduction

Complement (C) may be considered as the effector and amplification system of the humoral reaction (Opferkuch & Segerling 1977) and is furthermore activated by a number of naturally occurring polymers. C mediates a number of biological activities which may play an important role in host defense (Müller-Eberhard 1975). More precisely two complement activating pathways are known by which biologically active molecules are released:

- a) the classical pathway (CPW) which is initiated by a reaction of the first complement component (C_1) with immune complexes involving predominantly IgM or IgG,
- b) the alternative pathway (APW) activated predominantly by polysaccharides, bacterial lipopolysaccharides, bacterial cell walls and aggregated IgA (Götze & Müller-Eberhard 1971). They exercise either a protective or a deleterious effect on host tissues.

A participation of the C-system in the pathogenesis of periodontal disease has been suggested by several investigators (Mergenhausen, Tempel & Snyderman 1970, Allison, Schorlemmer & Bitter-Suermann 1976). Consequently C-components were detected in crevicular and pocket fluid (Shillitoe & Lehner 1972, Attström et al. 1975). Wilton, Renggli and Lehner (1977) demonstrated that polymorphonuclear leucocytes (PMN) migrating through the gingival sulcus carried C3b on their surface while PMNs derived from peripheral blood did not carry C3b on the respective receptors.

More recently, reports were published showing gram-positive

bacteria activate C by the APW (Tsai et al. 1977, Wilton 1977). Mutan, a water insoluble glucan synthesized by extracellular enzymes of S. mutans was also found to activate complement by the APW (Inai et al. 1976).

In the following experiment, cellular and extracellular fractions of A. viscosus Ny 1 were investigated for their ability to activate C by the APW.

4.2. Material and Methods.

Source of complement: Blood obtained after decapitation of healthy Osborne-Mendel rats was collected in ice-cooled plastic tubes and was immediately centrifuged (1200 g, 15 min, 4 C). Fresh C was used for each experiment.

Activating substances: Insoluble, pyrogen-free inulin was purchased from Sigma (Sigma London, Chemical Co Ltd). Cell walls (CW) of A. viscosus Ny 1 were prepared according to Reed (1972). Microbial cultures were grown in a modification (Bowden, Hardie & Fillery 1976) of the defined medium HB/6B. The extracellular fraction (ECHG) was obtained by precipitating the cell-free supernatant of the culture medium with ammonium sulfate at 80% saturation. The precipitate was then dissolved in 5 mM phosphate buffer pH 6.8 and extensively dialysed against the same buffer and then lyophilized (Burckhardt 1978). To prepare the broken cell supernatant (BCS) Actinomyces cells were extensively washed with saline and disrupted in a Braun MSK disintegrator (R. Braun, Melsungen GFR). Cell walls and insoluble particles were removed by high speed centrifugation (60 min, 105'000 g). To the supernatant 53 g/100 ml ammonium sulfate was added resulting in a approximately 80% saturation. The precipitate harvested by centrifugation was dissolved in buffered physio-

logical saline and dialysed against the same buffer before it was lyophilized. All steps were carried out at 4 C.

Chemicals: Gelatin veronal buffer (GVB) was prepared as described by Gewurz and Suyehira (1976). One ml of 2.0 M MgCl_2 and 0.3 M CaCl_2 stock solutions were added to 1 liter GVB when used for complement titration (GVB^{2+}).

By using ethylene glycol bis-amino tetraacetic acid buffer (EGTA, Fluka, CH-9470 Buchs), a strong chelator for Ca^{2+} , it was possible to selectively block the CPW at the C_1 level, thus allowing an estimation of the APW function.

EGTA was prepared as a 0.1 M stock solution in normal saline and brought to pH 7.4 as described by Fine et al. (1972).

Hemolytic assay: Sheep erythrocytes (SRBC) collected in Alsever solution were obtained from the clinic of veterinary medicine of the University of Zurich. Anti-sheep hemolysin was purchased from Difco (Difco Laboratories, Detroit Mich. USA). SRBC were sensitized (SRBC-Ab) according to the method of Mayer (1961). Complement activation was assessed as shown in figure 5.

Test system: Fifty μl of the test substances fivefold diluted in saline (1.6 $\mu\text{g/ml}$ -25 mg/ml final concentration) was incubated with 0.45 ml rat plasma previously chelated with 10 mM EGTA. After 30 min incubation in a water bath at 37 C, the reaction mixture was centrifuged to remove particles (2000 g, 10 min, 4 C). Thereafter the supernatant was collected and 50 μl of 0.1 M CaCl_2 and 0.05 M MgCl_2 in saline was added in order to reconstitute the hemolytic activity of plasma. Negative controls were prepared by the use of saline without test compounds. Inulin (10 mg/ml) was used as a positive control.

Indicator system: Probes of 25 μl were then twofold diluted in microtitration plates with 0.3 ml U-shaped wells (Flow

Laboratories Irvine, Scotland). Twenty-five μ l of SRBC-Ab and 125 μ l of GVB²⁺ were added and incubated for one hour in a tissue culture incubator at 37 C. The highest dilution where at least half of the SRBC were lysed was arbitrarily read as titer.

The results were expressed as percentage consumed hemolytic activity and were calculated by dividing the difference between the complement levels of challenged plasma and saline-treated controls with the saline-treated controls $\times 100$.

4.3. Results.

Activation of complement by the alternative pathway by several fractions of A. viscosus Ny 1 and by inulin is shown in figure 6.

Cell walls and inulin were the most active, followed by the extracellular fraction, whereas the broken cell supernatant was inactive.

Incubation of 0.0016-5 mg/ml of the cell wall fraction and 0.008-25 mg/ml of the extracellular fraction resulted in an increasing complement consumption of 5-87% and 5-75% respectively, which was evident by the decreasing hemolytic activity in the indicator system.

In all trials the positive control containing 10 mg/ml inulin depleted complement in the test system between 87-100%.

4.4. Discussion.

Activation of the CPW of complement is dependent on the presence of Mg^{2+} and Ca^{2+} , whereas activation of the APW requires only Mg^{2+} . By using EGTA, a strong chelator for Ca^{2+} Fine *et al.* (1972) introduced a method which allows to block

the CPW selectively and which does not interfere with C activation by the APW. Combined with the titration of the residual hemolytic C-activity in microtiter-plates the amount of complement activity consumed by the APW could be evaluated.

The following disadvantages however had to be considered:

(i) the estimation of the consumed C-activity in the indicator system was performed stepwise, in twofold dilution series. Therefore readings in the hemolytic indicator system were of limited accuracy. More precise readings could have been obtained by linear dilutions.

(ii) Furthermore, the sensitivity achieved was largely dependent on the plasma C-titer which determined the range of possible readings.

The results of the present investigation showed that in addition to cell walls an extracellular fraction of A. viscosus Ny 1 also activated the complement system by the APW. This is not an unique finding because it has been recently shown that various gram-positive bacteria (Greenblatt, Bockle & Schwab 1978, Wilson 1977, Tsai et al. 1977) as well as extracellular glucans and fructans (Inai et al. 1976, Leshner & Gerencser 1977) activated the APW.

There are observations which point to an important role of the C system in the etiology of periodontal disease. It has been shown that presumably all gram-negative bacteria and of course crude plaque (Schorlemmer, Bitter-Suermann & Allison 1977) are able to activate the APW. It was also demonstrated that activated complement components were present in sulcus fluid obtained from inflamed gingivae (Attström et al. 1974, Schenkein & Genco 1977).

In addition bacteria coated with C components were observed in subgingival samples from periodontal lesions (Nisengard

1977). It is well established that several breakdown products released in the sequence of C activation are mediators of early events in inflammation. Small fragments (C3a, C5a) resulting from the conversion of C3 and C5 exhibit chemotactic and anaphylactic activity (Opferkuch & Segerling 1977).

C3b the major conversion product of C3 is the most important opsonin. C3b binds to particles and thus facilitates their phagocytosis by PMN and macrophages. Furthermore it has been recently described by Schorlemmer, Bitter-Suermann and Allison (1977) that almost all known activators of the APW induced enzyme secretion by macrophages. Such enzymes, as e.g. collagenase or elastase are involved in the degradation of the acellular fraction of the connective tissue (Cimasoni et al. 1977).

5. The cytotoxic activity of the complement component C3a on rat fibroblasts in vitro.

5.1. Introduction.

Dental plaque or plaque components have a trigger function in the development of periodontal disease. An early event in periodontal destruction is massive collagen loss (Page & Schroeder 1976). Fragments of certain complement components, formed during complement activation, are probably involved in the initiation of the gingival inflammation as well as in the breakdown of the collagen fibrils, which are the structural basis of these tissues. Recently, Ferluga et al. (1976) described a further biological activity of C3a, the smaller of two active fragments of the third complement component. In absence of its inactivator, carboxypeptidase B, C3a destroyed human and murine tumor cells.

Besides the earlier mentioned loss of collagen, cytopathically changed fibroblasts were observed by Page and Schroeder (1976) in the early periodontal lesion. They speculated that this event was due to an interaction between lymphocytes and fibroblasts. Nevertheless a cytotoxic activity of C3a, which is regularly generated during the early stages of the inflammatory process, cannot be excluded. This hypothesis is furthermore supported by experiments of Allison, Schorlemmer and Bitter-Suermann (1976) and Tsai et al. (1977) who showed an antibody independent activation of the complement system by plaque or plaque constituents.

The present investigation describes the in vitro effect of guinea pig C3a in healthy fibroblasts, that had been isolated from fetal rat skin.

5.2. Material and Methods.

Preparation of C3a: Pure guinea-pig C3 (batch 40667/41677) was purchased from Cordis laboratories (Miami, Florida USA). Forty mg C3 were dissolved in 40 ml phosphate buffered saline (PBS), pH 7.6 and 0.4 mg trypsin (Sigma Chemical Co., London) were added. After 60 sec. 0.8 mg soybean trypsin inhibitor (STI, Sigma Chemical Co., London) was added to stop further cleavage. Thereafter the solution was acidified with 1.0 N HCl to pH 3.5, in order to dissociate the C3a fragment from cleaved C3-complex (Bokisch et al. 1969). The precipitate, containing C3b, was removed by centrifugation. The supernatant was concentrated to a final volume of 5 ml using an Amicon ultrafiltration cell equipped with an UM-05 membrane. The reaction products were preparatively chromatographed on a Sephadex G-100 column (90 x 2.5 cm) which was equilibrated with 0.15 M acetate-NaCl buffer pH 3.6 at a flow rate of 0.15 ml/h. Fractions of 2 ml were collected. A representative chromatogram is shown in figure 7. The first two peaks, presumably containing the trypsin-STI complex, were discarded. The fractions associated with the following peaks were pooled and concentrated to a final volume of 5 ml in an Amicon ultrafiltration cell using UM-05 membranes. All steps were performed at 4 C.

Target cells: All experiments were performed with 7 day old rat fibroblast monolayers (7th passage) grown in Dulbecco's minimal essential medium (DMEM), enriched with 15% fetal calf serum (FCS) in tissue culture dishes (Tissue Culture Cluster Dishes 3524, Costar, Cambridge USA). The cytotoxicity assay was performed without serum present.

Labelling of cells: Monolayers were labelled with ^3H -uridine (NEN Chemicals GmbH, D-6072 Dreieich, GFR), specific acti-

vity 5 Ci/mM, as described in 3.2.. Thereafter the cells were washed three times with PBS without Mg^{2+} or Ca^{2+} ions.

Cytotoxicity assay: The pH of the concentrated pools I and II were readjusted to the pH of the culture medium using 1 N NaOH. To each culture 0.5 ml of DMEM without serum and 0.1 ml of twofold dilutions of pool I and II diluted with medium were added. 0.1 ml of the elution buffer diluted with medium (1:2) served as medium control. Thereafter the cultures were incubated for 90 min at 37 C. Reactions were stopped by transferring the culture dishes into a refrigerator at 4 C.

Estimation of cytotoxic effects: The radioactivity of the culture supernatants was measured as described in 3.2. and was expressed as a percentage of the totally incorporated radioactivity. Means and standard deviations were calculated from 4 replications.

5.3. Results.

In table V percentage values of the means of released isotopes are shown. No cytotoxic effect was observed with dilutions 1:8 and 1:16 of pool I. In comparison to the control the 1:4 dilution contained a slightly increased amount of released isotope. The twofold dilution of pool I caused a clear cytotoxic effect.

Absolutely no cytotoxicity was observed with any dilution of pool II.

The control values of pool I and pool II differed slightly (separate culture plates). In order to compare both experiments the cytotoxicity of the control values was arbitrarily assigned 100% and also the experimental values transformed respectively.

The transformed values of table V are graphically presented

Table V

Mean $\pm$ standard deviation of percentage value of isotope released from rat fibroblasts upon incubation for 90 min with different concentrations of pool I and pool II of a guinea-pig C3a preparation. All experiments were performed with 7 day old monolayers of the 7th passage.

Dilution	pool I % of released isotope	pool II % of released isotope
1 : 16	16.5 $\pm$ 0.7	21.8 $\pm$ 0.9
1 : 8	16.2 $\pm$ 0.9	20.9 $\pm$ 1.2
1 : 4	18.9 $\pm$ 1.7	21.4 $\pm$ 0.8
1 : 2	36.2 $\pm$ 5.2	23.9 $\pm$ 0.3
Elution buffer:		
1 : 2	16.6 $\pm$ 1.0	22.5 $\pm$ 1.2

in figure 8.

5.4. Discussion.

The results of the present investigation demonstrated that fragments of C3 exerted a cytotoxic effect on rat fibroblasts in vitro. It was assumed that C3a was the toxic agent.

C3a releases histamin from rat mast cells (Johnson et al. 1975), has chemotactic activity for polymorphonuclear leucocytes (Ward et al. 1965) and mediates the contraction of smooth muscles in guinea-pig uterus (Dias da Silva et al. 1967). I tried in vain to demonstrate a reaction after injecting small amounts of pool I into rat skin. However, the rat is not a suitable animal for such experiments.

The above mentioned biological activities of C3a point to a possible role of this compound in local inflammatory reactions.

Recently Ferluga et al. (1976) showed that guinea-pig C3a was cytotoxic for various cells, including transformed human and murine cell lines. However, diploid cells were much more resistant towards the action of C3a. The cytopathic effect demonstrated in the present experiments was weak, however, comparable to the effect showed by Ferluga et al. (1976) with murine fibroblasts (L-cells).

The development of an acute exudative vasculitis and loss of perivascular collagen is characteristic for the initial lesion observed in gingivitis. In addition in the early lesion cytopathic changes in fibroblasts appeared (Page & Schroeder 1976). These histological changes should be interpreted as a result of complement activation. Oral micro-organisms have been shown to activate complement in the

absence of antibodies.

Vascular permeability was increased by the action of C3a and C5a. Such a reaction may be demonstrated in the presence of 10 mg of injected C5a (Lepow et al. 1970). The perivascular collagen loss in periodontal disease could be interpreted either as enzymatic degradation or alternatively by a negative balance between synthesized and resorbed collagen (Garant 1976). It is tempting to speculate that C3a might influence the overall rate of synthesis of collagen.

6. Complement-mediated cytotoxicity: Do rat fibroblasts carry receptors for antigens of *Actinomyces viscosus*?

6.1. Introduction.

The presence of immunoglobulins in the gingival fluid (Brandtzaeg 1965, Schenkein & Genco 1977) as well as in gingival tissues (Platt, Grosby & Dalbow 1970, Mackler et al. 1977) and penetration of soluble microbial antigens into the connective tissue of the gingiva (McDougall 1971, Tolo & Jensen 1975, Alfano, Chasens & Masi 1977, Ranney 1978) led to the hypothesis that reactions mediated by antibodies may participate in the destructive processes associated with periodontal disease.

This had already been postulated in 1929 by Hulin who showed severe periodontal inflammation in dogs hypersensitized with egg albumin after repeated topical application of this antigen to the gingiva.

Similar experiments were performed more recently by Rizzo and Mitchell (1966) in rabbits and by Ranney and Zander (1970) in squirrel monkeys with essentially the same results.

An impressive numeric decrease in fibroblasts was observed by Guggenheim and Schroeder (1974) after associating A. viscosus Ny 1 with gnotobiotic RIC-Sprague-Dawley rats previously sensitized with the same microorganism by intravenous injections. This reduction was associated with plasma cells dominating the infiltrate in the connective tissue of the gingiva. It was hypothesized by these authors that the loss of fibroblasts was the result of (i) antigen-antibody-complex-dependent cytotoxicity or (ii) complement-mediated cytotoxicity through antibodies reacting with antigenic components of A. viscosus Ny 1 adherent to the fibroblasts surfaces.

This model assumed that antigen receptors were present on the surfaces of these cells.

The following experiments investigated whether the latter mechanism was operative in vitro. For this purpose rat fibroblasts were incubated with antigen fractions of A. viscosus Ny 1 and then exposed to antiserum against the same organism. An attempt was then made to initiate a cytotoxic action by the addition of guinea-pig complement.

6.2. Material and Methods.

Complement, antisera and antigens: Lyophilized guinea-pig complement (complement activity: 200 CH₅₀ units) was purchased from the Institute for Medical Microbiology, St.Gallen (CH-9000 St.Gallen, Switzerland).

A non-absorbed, anti-rat-thymocyte serum from rabbits was used as a positive control.

A. viscosus Ny 1 was originally isolated by Dr. H. van der Hoeven from glucose fed rats.

An antiserum against whole cells of A. viscosus Ny 1 was raised in a rabbit. As demonstrated in microagglutination tests, it showed high antibody titers against the high molecular weight, watersoluble extracellular fraction (HMW-WS) as well as against the ammonium sulfate precipitate of the supernatant of broken cells (BCS-ASP) of A. viscosus Ny 1. Both above mentioned fractions served as antigens in the cytotoxicity tests described below.

HMW-WS was obtained by precipitating the cell free supernatant with ammonium sulfate at 80% saturation. The precipitate was dissolved in a 5 mM phosphate buffer at pH 6.8, extensively dialysed against the same buffer and then lyophilized (Burckhardt 1978). To prepare BCS-ASP, Actinomyces

cells were extensively washed with saline and then disintegrated in a Braun MSK disintegrator (B.Braun, Melsungen, GFR), cooled with CO_2 . The particulate fraction, including the cell walls, was removed by high speed centrifugation (1 h, 105'000 g). The supernatant was concentrated by precipitation with ammonium sulfate at 80% saturation.

The precipitate was redissolved in buffered physiological saline and dialysed against the same buffer. All steps were carried out at 4 C. Both fractions were lyophilized for storage.

Tissue culture: Diploid fibroblasts were derived from 18 to 20 day old RIC-Sprague-Dawley rat fetuses by finely mincing the skin followed by disaggregation with 0.25% trypsin (Tordaro & Green 1963).

The experiments were carried out with 7 day old confluent fibroblast monolayers between the 7th and 12th transfer after removal from primary explants. The cultures were maintained in Dulbecco's minimal essential medium (DMEM, Flow Laboratories Ltd., Irvine, Scotland) enriched with 15% heat inactivated fetal calf serum (FCS, Seromed, Munich, GFR). Cells to be used for cytotoxicity tests were cultivated in wells in disposable sterile polystyrene trays (Costar 3524, tissue culture cluster dishes, Digitana AG, CH-8810 Horgen, Switzerland). The cultures were incubated at 37 C in a humid atmosphere containing 5% CO_2 - 95% air.

After one week, confluent monolayers had developed. Cell density was then about 10^5 cells per cm^2 .

Labelling of cells: Confluent monolayers were washed twice in phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} , labelled for 1 hour with 1 ml DMEM containing 1 μCi (^3H -) uridine (specific activity 5 Ci/mM, New England Nuclear Chemicals GmbH, D-6072 Dreieich, GFR) and, before adding

any test agents, washed 3 times with PBS.

Puromycin treatment: To increase the susceptibility to cytotoxic effects cells were, first, incubated with 25 μg puromycin-di-HCl (Sigma London Chemicals Co. Ltd) per ml for 2 h at 37 C. Puromycin inhibits the reincorporation of released AIB (van den Berg & Betel 1971). Furthermore, all reaction mixtures to be described in figure 9 contained 10 μg of this compound per ml.

Cytotoxicity test: Test procedures applied in the cytotoxicity tests are schematically illustrated in a flow diagram (figure 9). The calculation of the cytotoxic effect was based on measurements of the radioactivity present in the supernatant and the activity remaining inside the fibroblasts at the end of the experimental period. To obtain a percentage value, the radioactivity measured in the supernatant was divided by the total of activity remaining in the cells plus that present in the supernatant.

The different treatments outlined in figure 9 were conducted in 2 sequential experiments. Means $\pm$ standard deviations were calculated from at least 4 cultures.

Measurement of radioactivity: 0.1 ml of the (^3H -) labelled sample was diluted in 10 ml Aquasol and the radioactivity was estimated in a LKB-Wallac 81'000 liquid scintillation counter. Automatic quench correction was by the internal standard method.

6.3. Results.

The results of this investigation are presented in figures 10 and 11.

A low spontaneous release of (^3H -)uridine was observed in both experiments as revealed by the medium control.

In the first experiment the guinea-pig complement, used in a final dilution of 1:10, showed essentially the same modest isotope release as the medium control. Therefore in the second experiment the complement control (figure 11) was excluded.

As a positive control, a cytotoxic effect was induced by the complement mediated action of an anti-rat-thymocyte serum. A clear, concentration dependent effect was observed, and cell damage was also microscopically detectable by distinct zones of cell lysis in the monolayer.

Exposure to 0.5 mg per ml of either fraction of A. viscosus Ny 1 (HMW-WS, BCS-ASP) followed by anti-A. viscosus Ny 1 serum and guinea-pig complement diluted 1:10 did not release more radioactivity than the medium controls. This definite absence of toxicity was independent of the antiserum dilution. The slightly increased value evident with BCS-ASP at an antiserum dilution of 1:10 (Fig. 11) had a large standard deviation and therefore was within the range of the controls.

6.4 Discussion.

Cytopathic alterations in resident fibroblasts and loss of the collagen fiber network of the marginal gingiva are attributes of early and established periodontal lesions (Page & Schroeder 1976). Cell-mediated hypersensitivity (Ivanyi, Wilton & Lehner 1972, Horton, Oppenheim & Mergenhagen 1972) or the cytotoxic as well as the immune complex-mediated types (Simpson & Avery 1974, Zachrisson 1968, Guggenheim & Schroeder 1974, Engel, Schroeder & Page 1978) are supposed to participate in its pathogenesis.

My experiments were based on the hypothesis that rat fibroblasts carry receptors for soluble antigens of A. viscosus Ny 1 on the cell surface. However, no complement-mediated cytotoxicity for rat fibroblasts was demonstrated either in the presence or absence of various antigens of A. viscosus Ny 1 and respective antibodies. This observation strongly suggests the absence of specific receptors for compounds of A. viscosus Ny 1 in high density on the surface of fibroblasts. A small number of receptors dispersed on the surface of these cells would most probably not have led to a measurable cytotoxic effect, even assuming that the complement system would have been activated. A strong lytic effect could however be induced by an anti-rat-thymocyte serum and complement, because this antiserum contained antibodies against a variety of antigens on the cell surface of the rat fibroblasts. Recently Engel, Schroeder & Page (1978) described receptors on human gingival fibroblasts for a crude antigen preparation of A. viscosus WVU/371. Because their preparation was a mixture of insoluble particles and soluble antigens, these experiments are not conclusive. The phagocytosis of particulate fragments is by no means evidence for the presence of genuine antigen receptors on the membranes of fibroblasts.

Phagocytosis by gingival fibroblasts does not seem to be relevant because microorganisms associated with periodontal disease are noninvasive. Furthermore the probability of a significant penetration of microbial particulate fractions through the gingival epithelium into the connective tissue is considered to be remote.

Of course the present results in no way rule out a possible role for antigen-antibody complexes in fibroblast cytotoxicity. If conditions would have been applied which would have allowed

the formation of soluble immune complexes i.e. by incubating cells, antibodies and antigens simultaneously, the possibility of observing a cytotoxic effect cannot be excluded. Such a mechanism, however, would depend on the presence of complement receptors on the surface of fibroblasts (Ueki et al. 1975). Further evidence for the participation of complement, activated by immune complexes, in the pathogenesis of periodontal disease has been provided by Fell, Coombs & Dingle (1966) and Hausmann (1974), who demonstrated complement-triggered activation of osteoclasts in vitro.

7. General discussion.

The purpose of the present study was to test mechanisms which, using an in vitro cytotoxicity assay, led to measurable alterations in rat fibroblast cultures. These processes could have imitated connective tissue breakdown during early stages of periodontal disease.

The most sensitive cytotoxicity tests are based on differences in the release of isotopes preliminarily incorporated. Above all, the experiments performed by Thelestam and Möllby (1975, 1976) pointed to test systems with non-metabolized aminoacids. A leakage test using the low-molecular weight alpha-amino-isobutyricacid was therefore adapted. A second tracer substance was however introduced (^3H -uridine), the formers use being restricted to short-time assays. Recently, experiments were published, in addition to a classical leakage test, evaluating alterations of the fibroblasts collagen metabolism (Engel et al. 1978, Narayanan & Page 1976). The present experiments were restricted to the estimation of the cell viability, comparing toxin induced with spontaneous isotope release.

Using less sensitive methods Baboolal et al. (1970) and Levine et al. (1973) showed marked inhibition of the growth of various cell-lines after exposition to human dental plaque extracts. These results were contradictory to those reported by Engel et al. (1978) who found, with essentially the same experimental arrangement, no cytotoxic and morphological alterations, when exposing human gingival fibroblasts to crude fractions of A. viscosus WVU/371. No cytotoxicity resulted from the incubation of rat fibroblasts with a watersoluble preparation of the extracellular slime of A. viscosus Ny 1.

The cellular fractions of the same bacteria however pathologically increased the isotope release after 1 hour exposure time only.

Mergenhausen, Tempel and Snyderman (1973) and Allison, Schorlemmer and Bitter-Suermann (1976) suggested the participation of the complement system in the pathogenesis of periodontitis. It has been furthermore shown that the complement cascade was activated by dental plaque and some of its microorganisms in systems freed of antibody. This mechanism, although operative in vitro, could be of importance to the induction of the acute inflammatory response in the gingiva. In my experiments the same process was induced by cell walls as well as by an extracellular, watersoluble fraction of A. viscosus Ny 1. Cytotoxic molecules and compounds are released by the complement activation. My results showed a weak cytotoxicity of the anaphylatoxin C3a, a low-molecular fragment of C3, for rat fibroblasts. So far, this effect was known only for transformed human and murine cell-lines (Ferluga et al. 1976). Specific receptors for A. viscosus were demonstrated on human gingival fibroblasts (Engel et al. 1978) and postulated on rat fibroblasts (Guggenheim & Schroeder 1974). Subsequent reactions, mediated by antibodies and complement and leading to cell injuries, were suggested. The last part of this thesis was dedicated to this problem. The results however led to the rejection of this interesting hypothesis. By no one of the fractions tested a cytotoxic reaction was evoked. The results of this study indicate a role for the complement system in the pathogenesis of periodontitis. This could be the mediation of unspecific effects like chemotaxis or mast cell degranulation with subsequent histamin release. Furthermore complement mediated cytotoxicity has to be considered.

It was however impossible to estimate its importance among all immune mechanisms attributed to participate in the pathogenesis of periodontal disease.

8. References.

- Aaronson, S.A., Todaro, G.J. 1968. Basis for the acquisition of malignant potential by mouse cells cultured in vitro. *Science* 162: 1024-1026.
- Abercrombie, M., Heaysman, J.E.M. 1954. Observations on the social behaviour of cells in tissue culture. *Exp. Cell Res.* 6: 293-306.
- Alfano, M.C., Chasens, A.I., Masi, C.W. 1977. Autoradiographic study of the penetration of radiolabelled dextrans and inulin through non-keratinized oral mucosa in vitro. *J. Periodontal Res.* 12: 368-377.
- Allison, A.C., Schorlemmer, H.U., Bitter-Suermann, D. 1976. Activation of complement by the alternative pathway as a factor in the pathogenesis of periodontal disease. *Lancet* ii: 1001-1004.
- Attström, R., Laurel, A.B., Laksson, U., Sjöholm, A. 1975. Complement factors in gingival crevice material from healthy and inflamed gingiva in humans. *J. Periodontal Res.* 10: 19-27.
- Baboolal, R., Mlinek, A., Powell, R.N. 1970. A study of the effects of gingival plaque extracts on cells cultured in vitro. *J. Periodontal Res.* 5: 248-254.
- Berg, van den, K.J., Betel, I.: Changes in aminoacid uptake in stimulated rat lymphocytes. *Proc. 6th leucocyte culture conference. Seattle 1971* pp 243-261 (Academic Press New York 1972).
- Bokisch, V.A., Müller-Eberhard, H.J., Cochrane, C.G. 1969. Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J. Exp. Med.* 129: 1109-1130.
- Bowden, G.H., Hardie, J.M., Fillery, E.D. 1976. Antigens from Actinomyces species and their value in identification. *J. dent. Res.* 55: Spec. Issue A, 192-204.
- Brandtzaeg, P. 1973. Immunology of inflammatory periodontal lesions. *Int. dent. J.* 23: 438-454.

Brandtzaeg, P., Kraus, F.W. 1965. Autoimmunity and periodontal disease. Odont. Tidskr. (Scand. J. dent. Res.) 73: 285-393.

Bunting, W.L., Liely, J.M., Owen, C.A. 1963. Radiochromium-labelled lymphocytes in the rat. Proc. Soc. Exp. Biol. 113: 370-374.

Burckhardt, J.J. 1978. Rat memory T lymphocytes: in vitro proliferation induced by antigens of Actinomyces viscosus. Scand. J. Immunol. 7: 167-172.

Carrel, A. 1912. On the permanent life of tissue outside of the organism. J. Exp. Med. 15: 516-528.

Ceccarini, C., Eagle, H. 1971. Induction and reversal of contact inhibition of growth by pH modification. Nature New Biol. 233: 271-273.

Christensen, H.N., Aspen, A.J., Rice, E.G. 1956. Metabolism in the rat of three aminoacids lacking alpha-hydrogen. J. Biol. Chem. 220: 287-294.

Christensen, H.N., Handlogten, M.E. 1968. Modes of mediated exodus of aminoacids from the Ehrlich ascites tumor cell. J. Biol. Chem. 243: 5428-5438.

Cimasoni, G., Ishikawa, I., Jaccard, F. Enzyme activity in the gingival crevice. In: Lehner, T. The borderland between caries and periodontal disease. pp 13-41 (Academic press London 1977).

Dias da Silva, W., Lepow, I.H. 1967. Complement as a mediator of inflammation. II. Biological properties of anaphylatoxin prepared with purified components of complement. J. Exp. Med. 125: 921-946.

Eagle, H. 1955. Nutrition needs of mammalian cells in tissue culture. Science 122: 501-504.

Eagle, H., Piez, K. 1962. The population-dependent requirements by cultured mammalian cells for metabolites which they can synthesize. J. Exp. Med. 116: 29-43.

Engel, D., Schroeder, H.E., Page, R.C. 1978. Morphological features and functional properties of human fibroblasts exposed to Actinomyces viscosus substances. Infect. Immun. 19: 287-295.

- Fell, H., Coombs, R., Dingle, J. 1966. The breakdown of embryonic (chick) cartilage and bone cultivated in the presence of complement sufficient antiserum. *Int. Arch. Allergy* 30: 146-176.
- Ferluga, J., Schorlemmer, H.U., Baptista, L.C., Allison, A.C. 1976. Cytolytic effects of the complement cleavage product C3a. *Br. J. Cancer* 34: 626-634.
- Fine, D.P., Marney, S.R., Colley, D.G., Sargent, J.S., Des Prez, R.M. 1972. C3 shunt activation in human serum chelated with EGTA. *J. Immunol.* 109: 807-809.
- Garant, Ph.R. 1976. Collagen resorption by fibroblasts. *J. Periodontol.* 47: 380-390.
- Gell, P.G.H., Coombs, P.R.A., Lachmann, P.J. 1975. Clinical aspects of immunology. 3rd edition (Blackwell, Oxford 1975).
- Gewurz, H., Suyehira, L. Complement. In: Rose, N.R., Friedman, H. Manual of clinical immunology pp 36-47 (American Society for Microbiology, Washington 1976).
- Gey, G.O., Coffman, W.D., Kubicek, M.T. 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* 12: 264-265.
- Götze, F., Müller-Eberhard, H.J. 1971. The C3 activator system: An alternative pathway of complement activation. *J. Exp. Med.* 134: 90s-108s.
- Greenblatt, J. Boackle, R.J., Schwab, J.H. 1978. Activation of the alternative complement pathway by peptidoglycan from streptococcal cell wall. *Infect. Immun.* 19: 296-303.
- Guggenheim, B., Schroeder, H.E. 1974. Reactions in the periodontium to continuous antigenic stimulation in sensitized gnotobiotic rats. *Infect. Immun.* 10: 565-577.
- Hausmann, E. 1974. Potential pathways for bone resorption in human periodontal disease. *J. Periodontol.* 45: 338-343.
- Hayflick, L., Moorhead, P.S. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 25: 585-621.
- Henney, C.S. 1973. Studies on the mechanism of lymphocyte mediated cytotoxicity: II. The use of various target cell mar-

kers to study cytolytic events. J. Immunol. 110: 73-84.

Holley, R.W., Kiernan, J.A. 1968. "Contact inhibition" of cell division in 3T3 cells. Proc. Nat. Acad. Sci. 60: 300-304.

Horton, J.E., Oppenheim, J.J., Mergenhagen, E. 1974. A role for cell-mediated immunity in the pathogenesis of periodontal disease. J. Periodontol. 45: 351-360.

Hoeven, van der, J.S. 1974. A slime-producing microorganism in dental plaque of rats, selected by glucose feeding. Caries Res. 8: 193-210.

Howell, A. jr. 1964. A filamentous microorganism isolated from periodontal plaque in hamsters. I: Isolation, morphology and general cultural characteristics. Sabouraudia 3: 81-92.

Howell, A. jr., Jordan, H.V., George, L.K., Pine, L. 1965. *Odontomyces viscosus*, gen. nov., spec. nov., a filamentous microorganism isolated from periodontal plaque in hamsters. Sabouraudia 4: 65-68.

Hulin, Ch. 1929. L'état anaphylactique et la pyorrhée. L'odontologie 67: 324-373.

Inai, S., Kazuyoshi, N., Shigeyuki, E., Keijro, K., Shozo, K., Misahi, A. 1976. Activation of the alternative complement pathway by water-insoluble glucans of *Streptococcus mutans*: The relation between their chemical structures and activating potencies. J. Immunol. 117: 1256-1260.

Ivanyi, L., Lehner, T. 1970. Stimulation of lymphocyte transformation by bacterial antigens in patients with periodontal disease. Arch. oral. Biol. 15: 1089-1096.

Ivanyi, L., Wilton, J.M.A., Lehner, T. 1972. Cell-mediated immunity in periodontal disease. Cytotoxicity, migration inhibition and lymphocyte transformation studies. Immunology 22: 141-145.

Johnson, A.R., Hugli, T.E., Müller-Eberhard, H.J. 1975. Release of histamine from rat mast cells by the complement peptides C3a and C5a. Immunology 28: 1067-1080.

Jordan, H.V., Keyes, P.H. 1964. Aerobic, grampositive, filamentous bacteria as etiologic agents of experimental peri-

odontal disease in hamsters. Arch. oral Biol. 9: 401-413.

Jordan, H.V., Fitzgerald, R.J., Stanley, H.R. 1965. Plaque formation and periodontal pathology in gnotobiotic rats infected with an oral actinomycete. Am. J. Pathol. 47: 1157-1167.

Lehner, T. In: Lehner, T. The borderland between caries and periodontal disease. Proceedings of a conference sponsored by the Royal Society of Medicine, 28 February 1977 (Academic Press, London 1977).

Lepow, I.H., Kretschmer, K.M., Patrick, R.A., Rosen, F.S., 1970. Gross and ultrastructural observations on lesions produced by intradermal injection of human C3a in man. Am. J. Pathol. 61: 13-20.

Leshner, R.J., Gerencser, V.F. 1977. Levan production by a strain of Rothia: Activation of complement resulting in cytotoxicity for human gingival cells. J. dent. Res. 56: 1097-1105.

Levine, M., Adams, R.L.P., Cowley, G.C. 1973. Effect of dental plaque extracts on mammalian cells in vitro. J. Periodontal Res. 8: 298-303.

Mackler, B.F., Frostead, K.B., Robertson, P.B., Levy, B.M. 1977. Immunoglobulin bearing lymphocytes and plasma cells in human periodontal disease. J. Periodontal Res. 12: 37-45.

Mayer, M.M. 1961. Complement and complement fixation. In: Kabat, E.A., Mayer, M.M. Experimental immunochemistry (2nd ed. Charles C. Thomas, Springfield, Ill. 1961).

McDougall, W.A. 1971. Penetration pathways of topically applied foreign protein into rat gingiva. J. Periodontal Res. 6: 89-99.

Mergenhausen, S.E., Tempel, T.R., Snyderman, R. 1970. Immunologic reactions and periodontal inflammation. J. dent. Res. 49: 256-261.

Metcalfe, S.M. 1971. Cell culture as a test system for toxicity. J. Pharm. Pharmacol. 23: 817-823.

Mlinek, A., Buchner, A., Hennig, S., Begleiter, A. 1974. Cytotoxic effects of soluble plaque extracts on cells in vitro. J. Periodontal Res. 9: 342-348.

- Müller-Eberhard, H.J. 1975. Complement. Amer. Rev. Biochem. 44: 697-724.
- Narayanan, A.S., Page, R.C. 1976. Biochemical characterization of collagen synthesized by fibroblasts derived from normal and diseased human gingiva. J. Biol. Chem. 251: 5464-5471.
- Nisengard, R.J. 1977. The role of immunology in periodontal disease. J. Periodontol. 48: 505-516.
- Opferkuch, W., Segerling, M. Biochemistry and biology of complement activation. In: Day, N.C., Good, R.A. Biological amplification systems in immunology, pp. 1-16 (Plenum, New York 1977).
- Page, R.C., Schroeder, H.E. 1973. Biochemical aspects of connective tissue alterations in inflammatory gingival and periodontal disease. Int. dent. J. 23: 455-469.
- Page, R.C., Schroeder, H.E. 1976. Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab. Invest. 33: 235-249.
- Platt, D., Crosby, R.G., Dalbow, M.H. 1970. Evidence for the presence of immunoglobulins in inflamed periodontal tissues. J. Periodontol. 41: 215-222.
- Raisz, L.G. 1965. Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. J. Clin. Invest. 44: 103-127.
- Ranney, R.R., Zander, H.A. 1970. Allergic periodontal disease in sensitized squirrel monkeys. J. Periodontol. 41: 12-21.
- Ranney, R.R. 1978. Immunofluorescent localization of soluble dental plaque components in human gingiva affected by periodontitis. J. Periodontal Res. 13: 99-108.
- Reed, M.J. 1972. Chemical and antigenic properties of the cell wall of Actinomyces viscosus (Strain T6). J. dent. Res. 51: 1193-1202.
- Rizzo, A.A., Mitchell, C.I. 1966. Chronic allergic inflammation induced by repeated deposition of antigen in rabbit gingival pockets. Periodontics 4: 5-10.
- Ruddle, N.H., Waksman, B.H. 1968. Cytotoxicity mediated by

soluble antigen and lymphocytes in delayed hypersensitivity. I. Characterization of the phenomenon. J. Exp. Med. 128: 1237-1254.

Sanford, K.K., Earle, W.R., Likely, G.D. 1948. The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229-246.

Schenkein, H.A., Genco, R.J. 1977. Gingival fluid and serum in periodontal diseases. Quantitative study of immunoglobulins, complement components and other plasma proteins. J. Periodontol. 48: 772-777.

Schorlemmer, H.U., Bitter-Suermann, D., Allison, A.C. 1977. Complement activation by the alternative pathway and macrophage enzyme secretion in the pathogenesis of chronic inflammation. Immunology 32: 929-940.

Schroeder, H.E., Münzel-Pedrazzoli, S., Page, R.C. 1973. Correlated morphometric and biochemical analysis of gingival tissue in early chronic gingivitis in man. Arch. oral Biol. 18: 899-923.

Schroeder, H.E., Lindhe, J. 1975. Conversion of stable established gingivitis in the dog into destructive periodontitis. Archs. oral Biol. 20: 775-782.

Shillioe, E.J., Lehner, T. 1972. Immunoglobulins and complement in crevicular fluid, serum and saliva in man. Archs. oral Biol. 17: 241- 247.

Simpson, D.M., Avery, B.E. 1974. Histopathologic and ultra-structural features of inflamed gingiva in the baboon. J. Periodontol. 45: 500-510.

Thelestam, M., Möllby, R. 1975. Sensitive assay for detection of toxin-induced damage to the cytoplasmic membrane of human diploid fibroblasts. Infect. Immun. 12: 225-232.

Thelestam, M., Möllby, R. 1976. Cytotoxic effects on the plasma membrane of human diploid fibroblasts - a comparative study of leakage tests. Med. Biol. 54: 39-49.

Todaro, G.J., Green, H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell. Biol. 17: 299-313.

Tolo, K., Jonsen, J. 1975. In vitro penetration of tritiated dextrans through rabbit oral mucosa. *Archs. oral Biol.* 20: 419-422.

Trudeau, D.L., Freier, E.F. 1974. Determination of calcium by atomic absorption spectrophotometry. In: Henry, R.J., Cannon, D.C., Winkelman, J.W. *Clinical chemistry, principles and technics*, pp 677-679 (Harper & Row, London 1974).

Tsai, C.C., Nilsson, U.R., McArthur, W.P., Taichman, N.S. 1977. Activation of the complement system by some gram-positive oral bacteria. *Archs. oral Biol.* 22: 309-312.

Ueki, A., Haguchi, Y., Hyodoh, F., Kimoto, R. 1975. The receptor sites for complement (C3) on human diploid fibroblasts. *Virchows Arch. B Cell Path.* 18: 101-107.

Ward, P.A., Cochrane, C.G., Müller-Eberhard, H.J. 1965. The role of serum complement in chemotaxis of leukocytes in vitro. *J. Exp. Med.* 122: 327-346.

Wigzell, H. 1965. Quantitative titrations of mouse μ -2 antibodies using ^{51}Cr -labelled target cells. *Transplantation* 3: 423-431.

Wilton, J.M.A. The function of complement in crevicular fluid. In: Lehner, T. *The borderland between caries and periodontal disease*, pp 223-247 (Academic Press, London 1977).

Wilton, J.M.A., Renggli, H.H., Lehner, T. 1977. The role of Fc and C3b receptors in phagocytosis by inflammatory polymorphonuclear leucocytes in man. *Immunology* 32: 955-961.

Yoshida, T., Cohen, S., Bigazzi, P.E., Kurasuji, T., Amsden, A. 1975. Inflammatory mediators in culture filtrates of Escherichia coli. Macrophage migration inhibitory, neutrophil chemotactic, lymphocyte mitogenic and fibroblast cytotoxic activities. *Am. J. Pathol.* 81: 389-400.

Zacchrisson, B.W. 1968. A histological study of experimental gingivitis in man. *J. Periodontal Res.* 3: 293-302.

9. Figures.

Figure 1: The relationship between seeding density ($\blacktriangle$: 10^2 cells/cm², $\blacksquare$: 10^3 cells/cm², $\bullet$: 10^4 cells/cm²), division rate (----) and cell number (——) in function of time. Fetal rat fibroblasts cultured in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Figure 2: The relationship between seeding density ($\blacktriangle$: 10^2 cells/cm², $\blacksquare$: 10^3 cells/cm², $\bullet$: 10^4 cells/cm²), division rate (----) and cell number (——) in function of time. Fetal rat fibroblasts cultured in DMEM supplemented with 15% FCS.

Figure 3: The relationship between seeding density ($\blacktriangle$: 10^2 cells/cm², $\blacksquare$: 10^3 cells/cm², $\bullet$: 10^4 cells/cm²), division rate (----) and cell number (——) in function of time. Fetal rat fibroblasts cultured in DMEM supplemented with 20% FCS.

Figure 4: Flow sheet for the leakage test used in direct cytotoxicity experiments. Hanks' BSS: Hanks' balanced salt solution. AIB: amino-isobutyric acid. FCS: fetal calf serum.

Figure 5: Measurement of consumed complement activity in rat plasma activated by the alternative pathway.

Figure 6: Consumed activity of complement after activation of the alternative pathway in EGTA-chelated rat plasma with fractions of A. viscosus Ny 1 ($\star$ ECHG: extracellular heteroglycan, $\blacksquare$ CW: cell walls, $\bullet$ BCS: broken cell supernatant;

▲ inulin: positive control). The reaction mixtures were incubated for 30 min at 37 C. Complement titers were estimated in a hemolytic system in microtiter plates after reconstitution of the classical pathway by adding 0.01 M CaCl_2 and 0.005 M MgCl_2 to the rat plasma.

Figure 7: Elution diagram after fractionation of C3 on Sephadex G-100. Elution with 0.15 M acetate buffered NaCl, pH 3.6. Flow rate: 15 ml/h. Fractions of 2 ml were collected. Pool I showed cytotoxic activity.

Figure 8: Cytotoxic effect on fetal rat fibroblasts of various dilutions of pool I (■) and pool II (●) of a guinea-pig C3a preparation. The results represent mean $\pm$ standard deviation of quadruplicates and are percentages of the elution buffer control. Incubation time: 90 min at 37 C.

Figure 9: Flow sheet for the test of antibody-complement complex mediated cytotoxicity. Five treatments were included. PBS: Phosphate buffered saline, pH 7.3, without Ca^{2+} and Mg^{2+} . RPMI 1640 was purchased from Flow Laboratories Ltd, Irvine, Scotland. BCS-ASP: Broken cell supernatant, HMW-WS: water-soluble fraction of the extracellular slime of A. viscosus Ny 1.

Figure 10: Release of ^3H -uridine from fibroblasts either untreated (O), complement treated (□), anti-rat-thymocyte serum treated (●) or exposed to 0.5 mg per ml of HMW-WS and anti-A. viscosus Ny 1 serum (■). The cytolytic action was induced by treatment with complement diluted 1:10. Data represent mean $\pm$ standard deviation of at least 4 replications.

Figure 11: Release of ^3H -uridine from fibroblasts, either untreated (O), anti-rat-thymocyte serum treated (●), or exposed to 0.5 mg/ml BCS-ASP and anti-A. viscosus Ny 1 serum (▲). The cytolytic action was induced by treatment with complement, diluted 1:10. Data represent mean $\pm$ standard deviation of 4 replications.

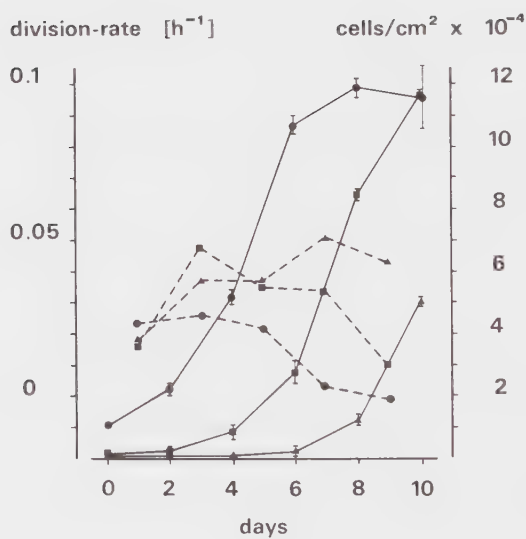


Figure 1

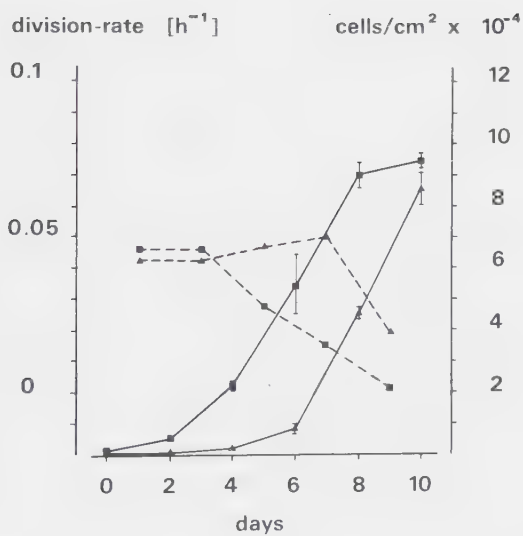


Figure 2

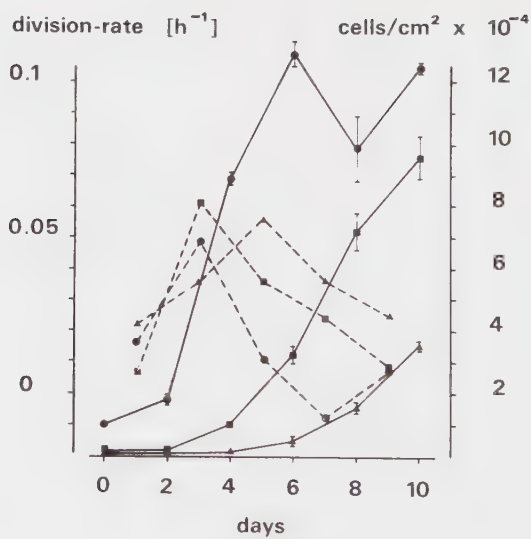


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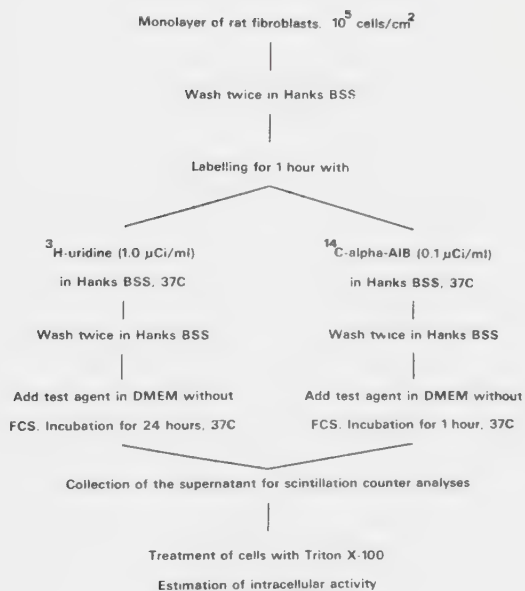


Figure 4

I. TEST SYSTEM

Assay for alternative pathway activation: Addition of EGTA blocks classical pathway

Bacterial fraction + Complement + EGTA = APW activation

II. INDICATOR SYSTEM

Assay for remaining not converted complement activity: Addition of CaCl_2 restores classical pathway activation

Sensitized sheep erythrocytes + Complement + CaCl_2 = CPW activation

Figure 5

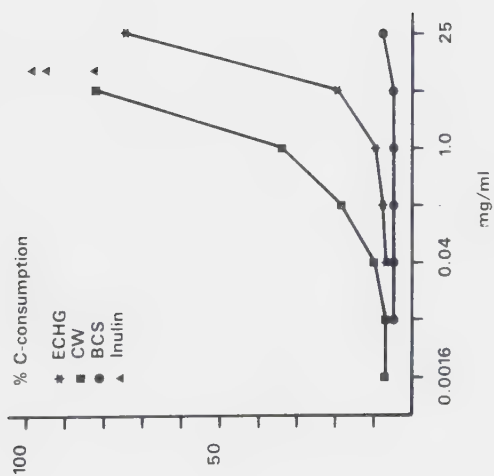


Figure 6

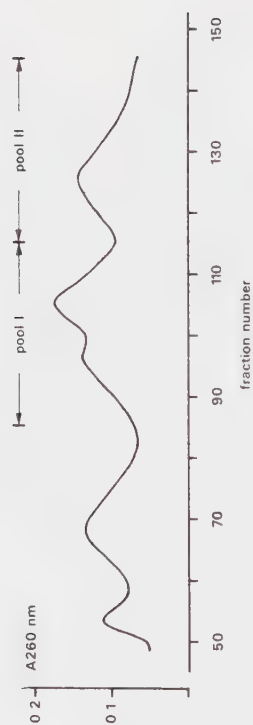


Figure 7

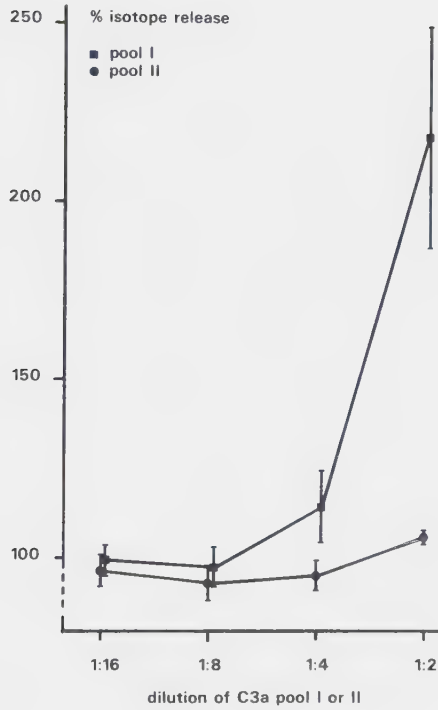


Figure 8

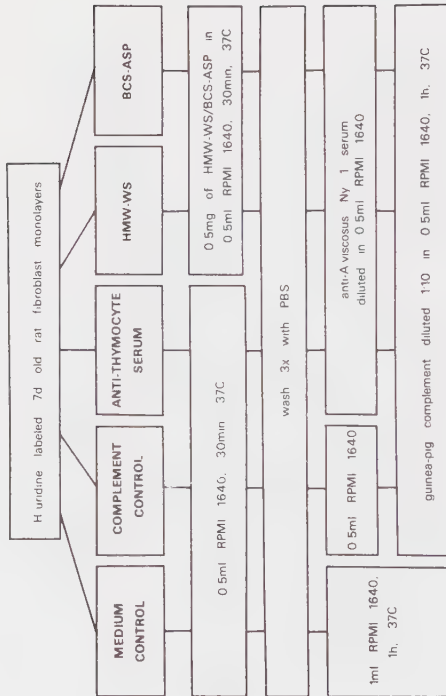


Figure 9

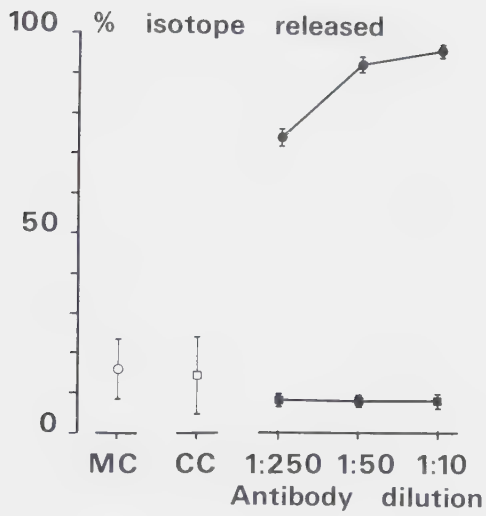


Figure 10

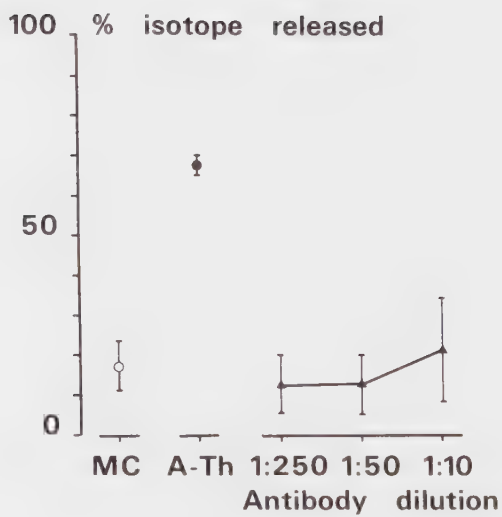


Figure 11

10. Summary.

A cytotoxicity test system using 14 alpha-aminoisobutyric-acid or 3 H-uridine labelled fetal rat fibroblasts was established and applied to the study of possible immune mechanisms causing injury to fibroblasts. In particular the following problems were approached:

- I. The direct cytotoxicity of fractions from A. viscosus Ny 1 was investigated.
- II. The activation of the complement cascade by fractions of A. viscosus Ny 1 by the alternative pathway was measured.
- III. The cytotoxicity of C3a, a cleavage product of C3 liberated while activating the complement system, was tested.
- IV. Antibody-complement-mediated cytotoxicity was investigated after preincubating fibroblast monolayers with soluble antigens from A. viscosus Ny 1.

The most important results of this in vitro study were:

1. Even small amounts of the cell wall (CW) and broken cell supernatant (BCS) fractions were cytotoxic whereas the extracellular heteroglycan (ECHG) affected the release of isotope only in high concentrations. Cytotoxicity was already detectable after 1 h incubation time.
2. In cubation of 0.0016-5 mg/ml of the CW and 0.008-25 mg/ml of the ECHG fraction resulted in an increasing complement consumption of 5-87% and 5-75% respectively. The BCS fraction was ineffective.
3. After enzymatic cleavage of C3 and subsequent separation of C3b and C3a on a Sephadex G-100 column, two C3a pools were obtained. After 90 min incubation time pool I caused a clear cytotoxic effect.

4. Preincubation of fibroblasts with the high molecular weight, water-soluble, extracellular fraction or the ammonium sulfate precipitate of disrupted *Actinomyces* cells and subsequent exposure to a rabbit anti-A. viscosus Ny 1 serum and guinea-pig complement had no cytotoxic effect for rat fibroblasts. This finding indicates the absence of receptors for soluble antigens of A. viscosus Ny 1 on these cells.

The results of this thesis imply a role for the complement system in the pathogenesis of periodontitis.

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CURRICULUM VITAE

Am 13.Mai 1947 wurde ich in Zürich geboren und besuchte dort die Primar- und Sekundarschule.

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